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(71) Applicants: CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). THE STATE OF OREGON acting by and through THE OREGON STATE BOARD OF HIGHER EDUCATION on behalf of THE UNIVERSITY OF OREGON [US/US]; Susan Campbell Hall, University of Oregon Campus, Eugene, OR 97403-1229 (US).

(72) Inventors: DRUMMOND, Robert, J. ; 2920 Cindy Court, Richmond, CA 94803 (US). BLOCH, Will ; 421 Liberty Street, El Cerrito, CA 94530 (US). MATTHEWS, Brian, W. ; 3815 Spring Boulevard, Eugene, OR 94705 (US). TOY, Pamela, L. ; 363 Somerset, Oakland, CA 94611 (US). NICHOLSON, Henry, H. ; 1366 Lawrence Street, Eugene, OR 97405 (US).

(74) Agents: MANDEL, Saralynn et al.; Ciotti & Murashige, Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

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(54) Title: PROCARYOTIC XYLOSE ISOMERASE MUTEINS AND METHOD TO INCREASE PROTEIN STABILITY

(57) Abstract

Xylose isomerase (XI) muteins useful in the conversion of glucose to fructose or xylose to xylulose are obtained in usable amounts by protein structural and recombinant DNA methods, including x-ray crystallography, cloning, computer graphic modeling and site-directed mutagenesis and expression of the bacterial DNA sequences encoding native procaryotic xylose isomerase. These native sequences are altered to encode the xylose isomerase muteins having improved catalytic function and/or thermostability, and/or a lowered pH optimum. A method for predicting protein-stabilizing amino acid substitutions is also provided.

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What is claimed is:

1. A method for increasing the stability of a protein comprising substituting an amino acid at a preselected substitution site in the protein, said substitution site having phi and psi backbone conformational angles in the range of phi = -40° to -90° when psi = 0° to -60°, or in the range of phi = -40° to -95° when psi = 120° to 180° and capable of accomodating said amino acid without disruption of the three-dimensional structure of the protein such that introduction of said amino acid decreases the configurational entropy of unfolding of said protein.

2. The method of Claim 1 wherein said preselected substitution site is any amino acid residue except proline and the amino acid introduced at said site is proline, and said method further comprises the step of determining the phi and psi values of the amino acid residue in the amino acid sequence of the protein immediately preceding the side of said proline substitution, such that if the psi value of the preceding amino acid residue is between 0° and -90° then the substitution site must have phi and psi values in the range of phi = -40° to -90° when psi = 0° to -60°, but if the psi value of the preceding amino acid residue is not between 0° and -90° the the substitution site may have phi and psi values either in the range of phi = -40° to -90° when psi = 0° to -60°, or in the range of phi = -40° to -95° when psi = 120° to 180°.

3. The method of Claim 1 wherein said preselected substitution site is a glycine amino acid residue and the amino acid introduced is any amino acid having a β carbon atom or branched β carbon atom.

4. A method for increasing the stability of a protein comprising substituting a glycine amino acid residue having a

negative phi angle with an alanine to decrease the configurational entropy of unfolding of the protein.

5. A method for selecting substitution sites suitable for introduction of amino acids in a protein such that introduction of said amino acids increases the stability of the protein, comprising the steps of:

a) determining from the crystallographic structure of a protein the backbone conformational angles phi and psi of said protein;

b) screening said phi and psi angles determined in step a) to identify potential substitution sites in said protein having conformational phi and psi angles in the range of phi = -40° to -90° when psi = 0° to -60°, or in the range of phi = -40° to -95° when psi = 120° to 180° for introduction of said amino acids; and

c) examining a structural model of the protein to determine from the potential substitution sites identified in step b) substitution sites that will accomodate substitution of an amino acid without disruption of the three-dimensional structure of the protein, whereby substitution of said substitution site results in a decrease in the configurational entropy of unfolding of the protein.

6. The method of Claim 5 wherein the amino acid to be substituted into said substitution site is proline, and the step of screening of step b) comprises the additional substep of determining whether the amino acid residue preceding the potential substitution site identified in step b) has psi angles between 0° and -90°, and if so then the step c) of examining comprises the substep of determining a substitution site having phi and psi angles in the range phi = -40° to -90° when psi = 0° to -60°.

7. Streptomyces rubiginosus, (S. rubiginosus), xylose isomerase mutein having a change in at least one position in the native amino acid sequence at a position equivalent to a native amino acid residue selected from the group consisting of $Lysine_{183}$, $Lysine_{289}$, $Histidine_{54}$, $Histidine_{220}$, $Methionine_{223}$, $Arginine_{140}$, $Tryptophan_{16}$, $Tryptophan_{137}$, $Phenylalanine_{94}$, $Glycine_{146}$, $Glycine_{166}$, $Glycine_{197}$, $Glycine_{219}$, $Glycine_{231}$, $Glycine_{248}$, $Glycine_{298}$, $Glycine_{305}$, $Glycine_{369}$, $Leucine_{15}$, $Alanine_{29}$, $Alanine_{33}$, $Asparagine_{107}$, $Arginine_{109}$, $Glycine_{146}$, $Valine_{151}$, $Glycine_{189}$, $Leucine_{192}$, Glutamic $acid_{207}$, $Arginine_{259}$, $Threonine_{342}$, $Arginine_{354}$, $Glycine_{369}$, Aspartic $acid_{28}$, $Arginine_{32}$, $Serine_{64}$, $Valine_{218}$, $Arginine_{292}$, $Isoleucine_{252}$, Aspartic $acid_{9}$, $Glutamine_{21}$, $Alanine_{29}$, $Arginine_{32}$, Glutamic $acid_{38}$, $Leucine_{46}$, Aspartic $acid_{56}$, $Leucine_{58}$, $Valine_{127}$, $Threonine_{133}$, $Alanine_{136}$, $Arginine_{177}$, $Isoleucine_{180}$, $Leucine_{193}$, $Leucine_{211}$, $Asparagine_{227}$, $Glutamine_{234}$, $Alanine_{238}$, $Leucine_{246}$, $Arginine_{284}$, $Arginine_{308}$, $Leucine_{311}$, $Arginine_{316}$, $Leucine_{335}$, $Valine_{362}$, $Methionine_{370}$, $Leucine_{375}$, $Leucine_{383}$, $Glutamine_{21}$, $Asparagine_{92}$, $Asparagine_{107}$, $Asparagine_{185}$, $Asparagine_{227}$, $Glutamine_{234}$, $Glutamine_{256}$, $Asparagine_{309}$, $Glutamine_{377}$, $Tryptophan_{270}$, $Glycine_{146}$, $Phenylalanine_{320}$, $Histidine_{382}$, Glutamic $acid_{337}$, $Arginine_{109}$, $Glycine_{189}$, Glutamic $acid_{144}$, $Glycine_{251}$, $Glycine_{225}$, $Alanine_{366}$, $Valine_{98}$, $Glutamine_{249}$, $Glycine_{219}$, Glutamic $acid_{207}$, Aspartic $acid_{163}$, Aspartic acid 57, Glutamic acid 186; Glutamic acid 141, Glutamic $acid_{221}$, Aspartic $acid_{287}$; $Arginine_{177}$; and Aspartic $acid_{345}$.

8. The S. rubiginosus xylose isomerase mutein of Claim 7 wherein the change is in the lysine amino acid residue equivalent to Lys_{183} and said change is substitution by an amino acid selected from the group consisting of Arg, Gln, Asn, Asp, Glu, Ser, Thr, His, Tyr, Ala, Val, Leu and Ile; or

the change is in the lysine amino acid residue equivalent to Lysine289 and said change is substitution by an amino acid selected from the group consisting of Arg, Gln, Asn, Asp, Glu, Ser Thr, His, Tyr, Ala, Val, Leu and Ile; or

the change is in the histidine amino acid residue equivalent to His_{54} and said change is substitution by an amino acid selected from the group consisting of Gln, Glu, Asn, Asp, Ser, Thr, Ala, Val, and Tyr; or

the change is in the histidine amino acid residue equivalent to His_{220} and said change is substitution by an amino acid selected from the group consisting of Gln, Glu, Asn, Asp, Ser, Thr, Ala, Val, and Tyr; or

the change is in the methionine amino acid residue equivalent to Met_{223} and said change is subsitution by an amino acid selected from the group consisting of Gly, Ala, Val, Leu, Ile, Phe, Tyr, Gln, and Asn; or

the change is in the arginine amino acid residue equivalent to Arg_{140} and said change is substitution by an amino acid selected from the group consisting of Gln, Asn, Glu, Asp, Ile, Leu, Ala, Val, and Tyr; or

the change is in the tryptophan amino acid residue equivalent to Trp_{16} and said change is substitution by an amino acid selected from the group consisting of Asn, Gln, Ser, Thr, Gly, Ala, Val, Leu, Ile, Tyr, Phe, and His; or

the change is in the tryptophan amino acid residue equivalent to Trp_{137} and said change is substitution by an amino acid selected from the group consisting of Asn, Gln, Ser, Thr, Gly, Ala, Val, Leu, Ile, Tyr, Phe, and His; or

the change is in the phenylalanine amino acid residue equivalent to Phe94 and said change is substitution by an amino acid selected from the group consisting of Thr, Ser, His, Val, Gly, Ala, Ile, Leu, Asn, and Gln; or

the change is substitution of the glycine amino acid residue equivalent to Gly_x where x is selected from the group consisting of residues 146, 166, 197, 219, 231, 248, 298, 305 and 369, and said Gly substituted with an amino acid other than glycine; or

the change is substitution by proline in the amino acid residue equivalent to an amino acid residue selected from the group consisting of Leu_{15}, Asp_{28}, Ala_{29}, Arg_{32}, Ala_{33}, Ser_{64}, Asn_{107}, Arg_{109}, Gly_{146}, Val_{151}, Gly_{189}, Leu_{192}, Glu_{207}, Val_{218}, Ile_{252}, Arg_{259}, Arg_{292}, Thr_{342}, Arg_{354}, Gly_{369}, Arg_{177}, and Asp_{345}; or

the change is double substitutions of cysteine in the amino acid residues equivalent to pairs of amino acid residues selected from the group consisting of Trp_{270} and Gly_{146}, Phe_{320} and His_{382}, Glu_{337} and Arg_{109}, Gly_{189} and Glu_{144}, Gly_{251} and Gly_{225}, Ala_{336} and Val_{98}, Gln_{249} and Gly_{219}, and/or Glu_{207} and Asp_{163}; or

the change is substitution by tyrosine in the amino acid residues equivalent to an amino acid residue selected from the group consisting of Asp_9, Gln_{21}, Ala_{29}, Arg_{32}, Glu_{38}, Leu_{46}, Asp_{56}, Leu_{58}, Val_{127}, Thr_{133}, Ala_{136}, Arg_{177}, Ile_{180}, Leu_{193}, Leu_{211}, Asn_{227}, Gln_{234}, Ala_{238}, Leu_{246}, Arg_{284}, Arg_{308}, Leu_{311}, Arg_{316}, Leu_{335}, Val_{362}, Met_{370}, Leu_{375} and Leu_{383}; or

the change is substitution by phenylalanine in the amino acid residue equivalent to an amino acid residue selected from the group consisting of Leu_{46}, Asp_{56}, Leu_{58}, Thr_{133}, Ala_{136}, Ile_{180}, Leu_{193}, Leu_{211}, Asn_{227}, Gln_{234}, Ala_{238}, Leu_{246}, Leu_{311}, Leu_{335}, Val_{362}, Met_{370}, Leu_{375} and Leu_{383}; or

the change is substitution by tryptophan in the amino acid residue equivalent to Asn227; or

the change is substitution by an amino acid residue selected from the group consisting of Ala, Val, Leu, Ile, Ser, Thr, His, Tyr, Lys, Arg, Met and Pro in the amino acid residue equivalent to an amino acid residue selected from the group consisting of Gln_{21}, Asn_{92}, Asn_{107}, Asn_{185}, Asn_{227}, Gln_{234}, Gln_{256}, Asn_{309}, and Gln_{377}; or

the change is in the aspartic acid amino acid residue equivalent to Asp_{57} and said change is substitution by an amino acid selected from the group consisting of Lys, Arg, Gly, Ala, Gln, Asn, Thr and Ser; or

the change is in the glutamic acid amino acid residue equivalent to Glu_{186} and said change is substitution by an amino acid selected from the group consisting of Lys, Arg, Gly, Ala, Gln, Asn, Thr and Ser; or

the change is substitution of the aspartic acid amino acid residue equivalent to Asp_{57} and said substitution is with an amino acid other than aspartic acid or glutamic acid; or

the change is substitution in the glutamic acid amino acid residue equivalent to Glu_{186} and said change is substitution by an amino acid other than aspartic acid or glutamic acid; or

the change is substitution by glutamine in the glutamic acid amino acid residue equivalent to Glu_{221}; or

the change is substitution by glutamine in the glutamic acid amino acid residue equivalent to Glu_{141}.

9. A nucleic acid encoding the xylose isomerase of Claim 7 or 8 said nucleic acid being substantially free of nucleic acid that does not encode the xylose isomerase of Claim 7 or 8.

10. An expression vector for mutant procaryotic xylose isomerase which comprises the nucleic acid of Claim 9 operably linked to control sequences compatible with a host cell.

11. A method for enhancing the conversion of glucose to fructose and xylose to xylulose which comprises exposing an effective amount of the xylose isomerase mutein of Claim 7 or 8 to glucose and xylose, respectively.

12. The xylose isomerase mutein of Claim 7 wherein the expressed xylose isomerase exhibits a change in one or more of the characteristics of chemical stability, k_{cat_f}, k_{cat_r}, K_S, K_P, temperature stability, specific activity and a lowered pH optimum of the isomerase, as compared to the reference xylose isomerase.

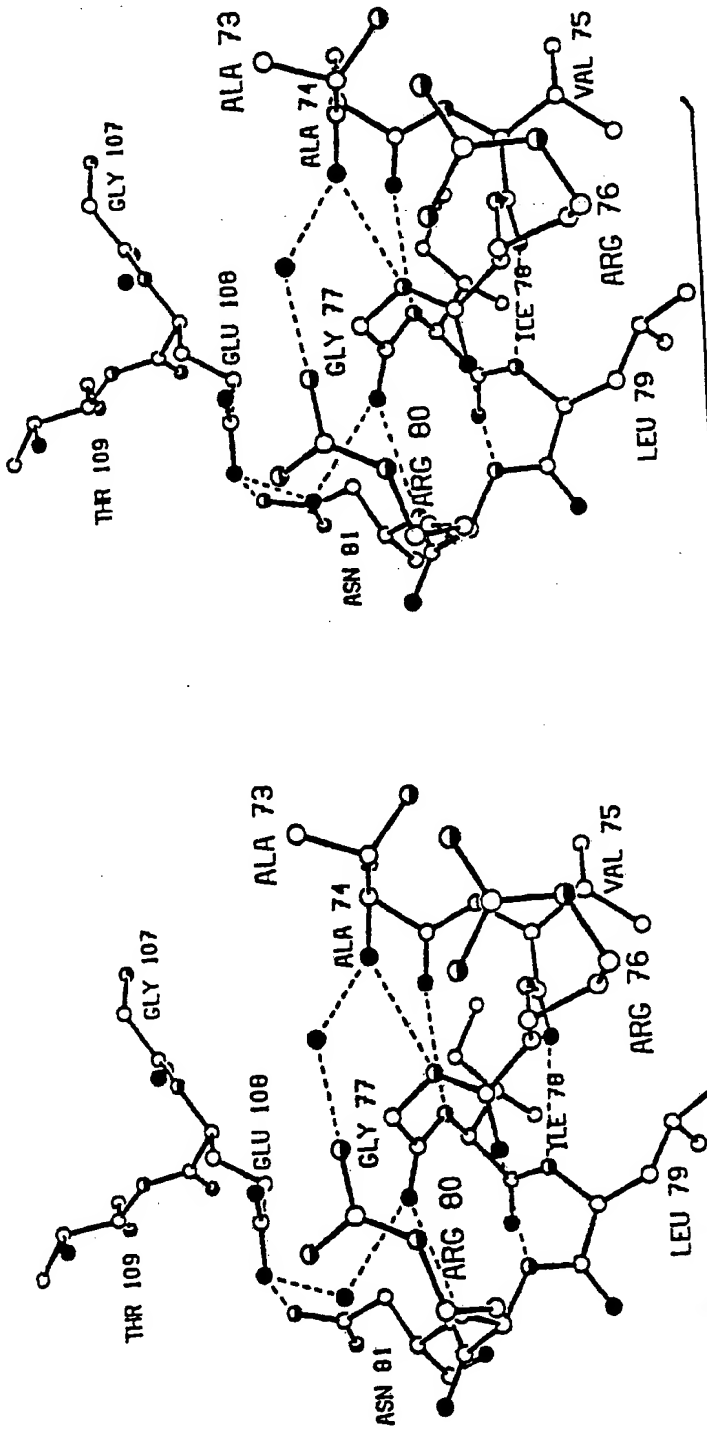

ALA 73
ALA 74
VAL 75
ARG 76
GLY 77
ILE 78
LEU 79
ARG 80
ASN 81
GLU 108
GLY 107
THR 109

FIG. 1A

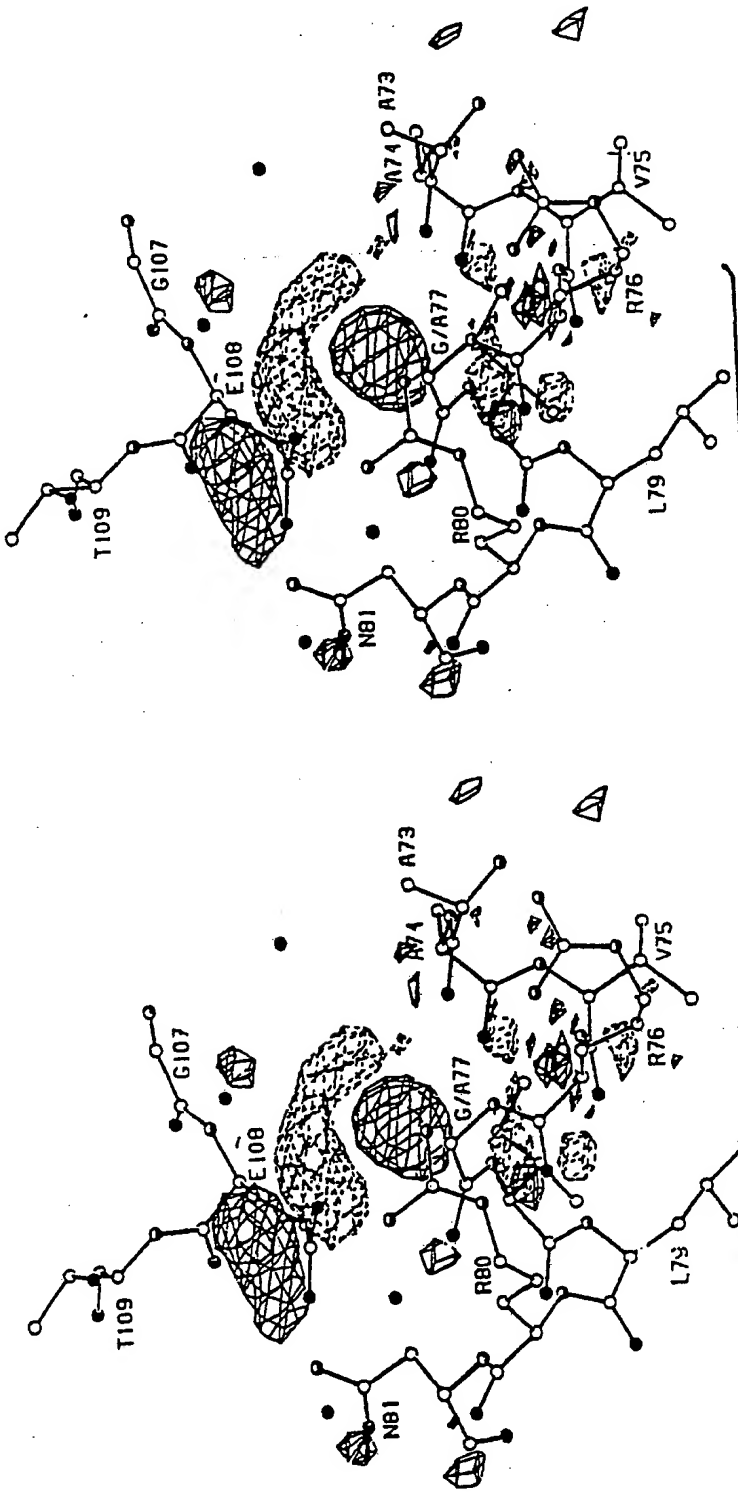

A73
N74
V75
G107
R76
G/A77
E108
T109
R80
L79
N81
A73
N74
V75
G107
R76
G/A77
E108
T109
R80
L79
N81

FIG. 1B

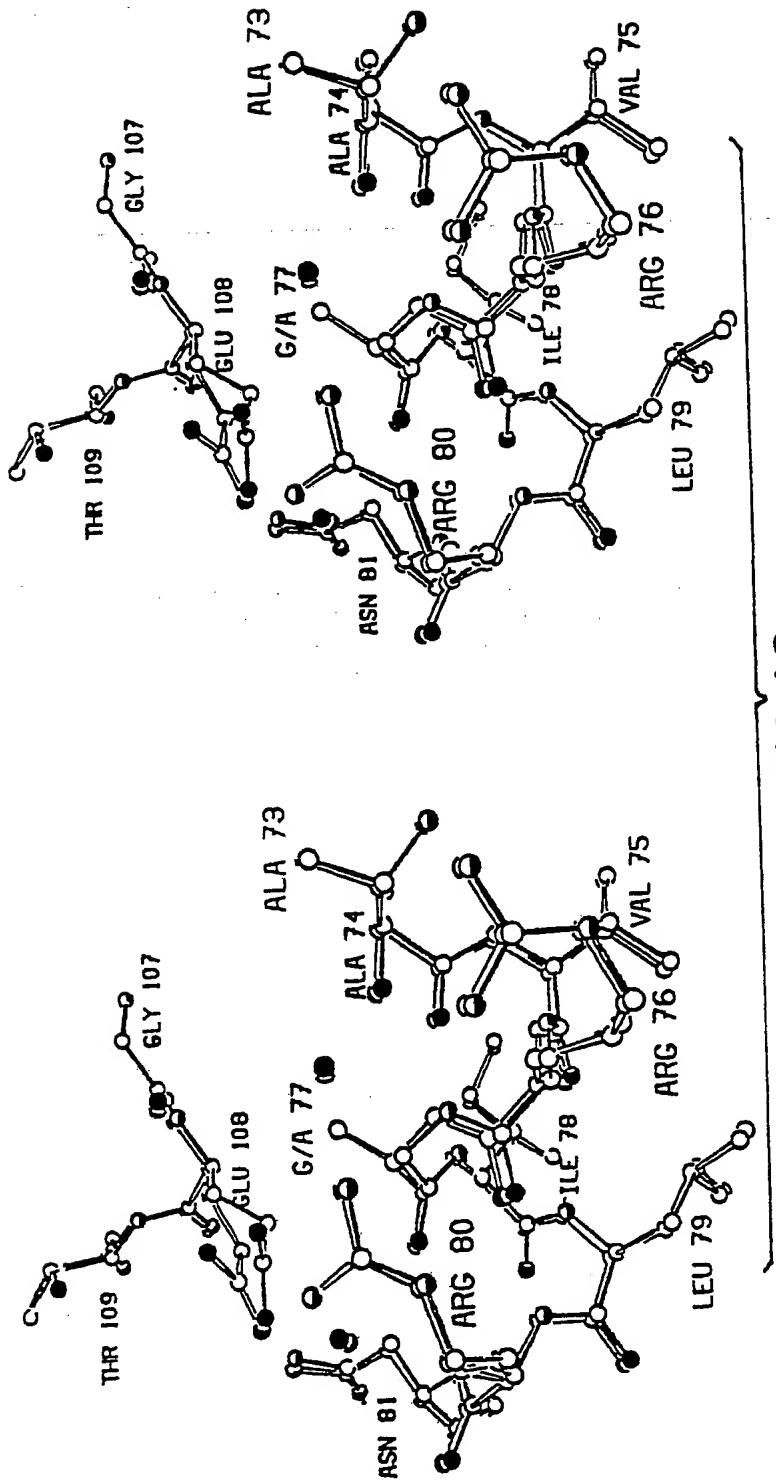

ALA 73
ALA 74
VAL 75
ARG 76
G/A 77
ILE 78
LEU 79
ARG 80
ASN 81
GLY 107
GLU 108
THR 109

FIG. IC

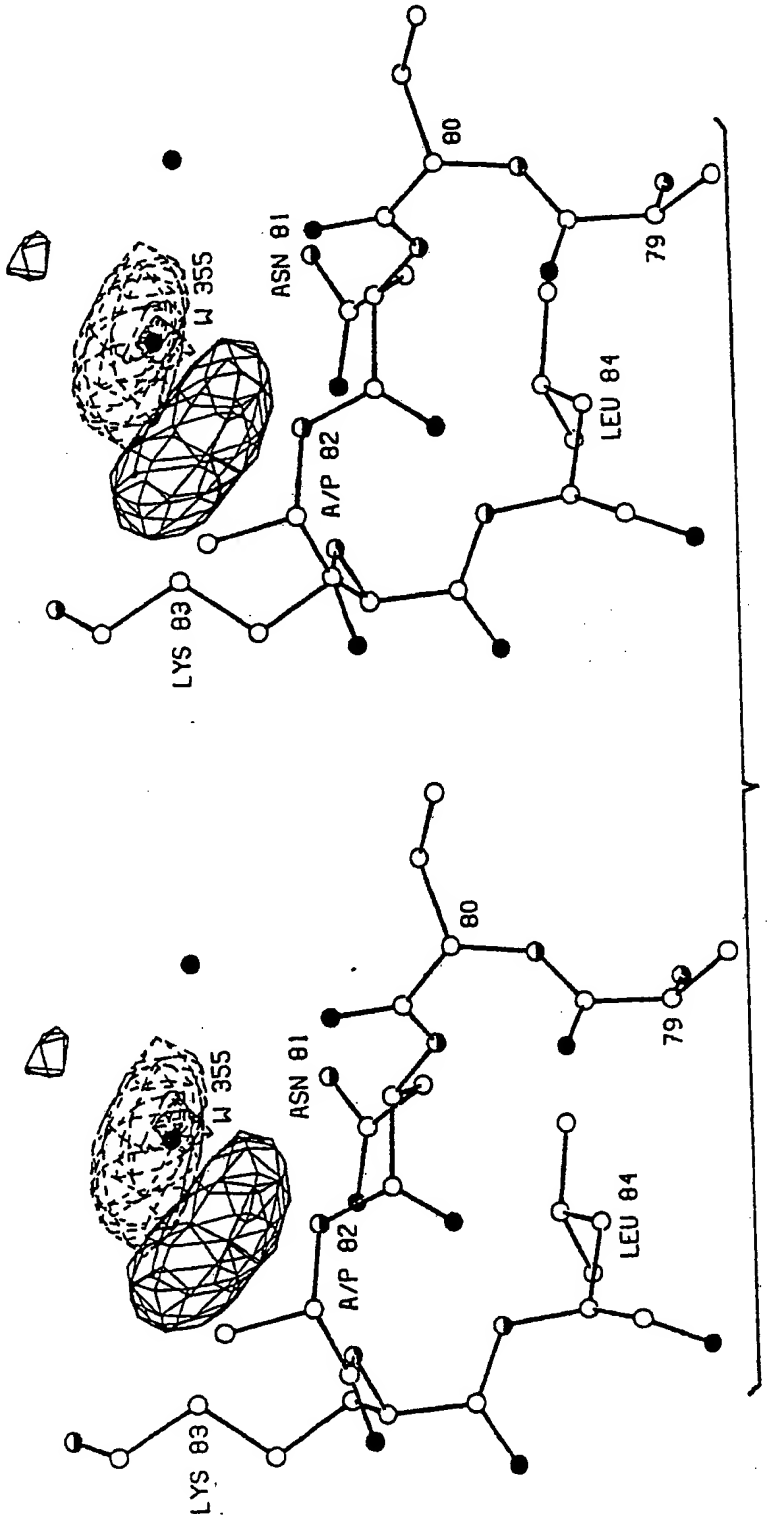

80
79
ASN 81
W 355
LEU 84
A/P 82
LYS 83
80
79
ASN 81
W 355
LEU 84
A/P 82
LYS 83

FIG. 2A

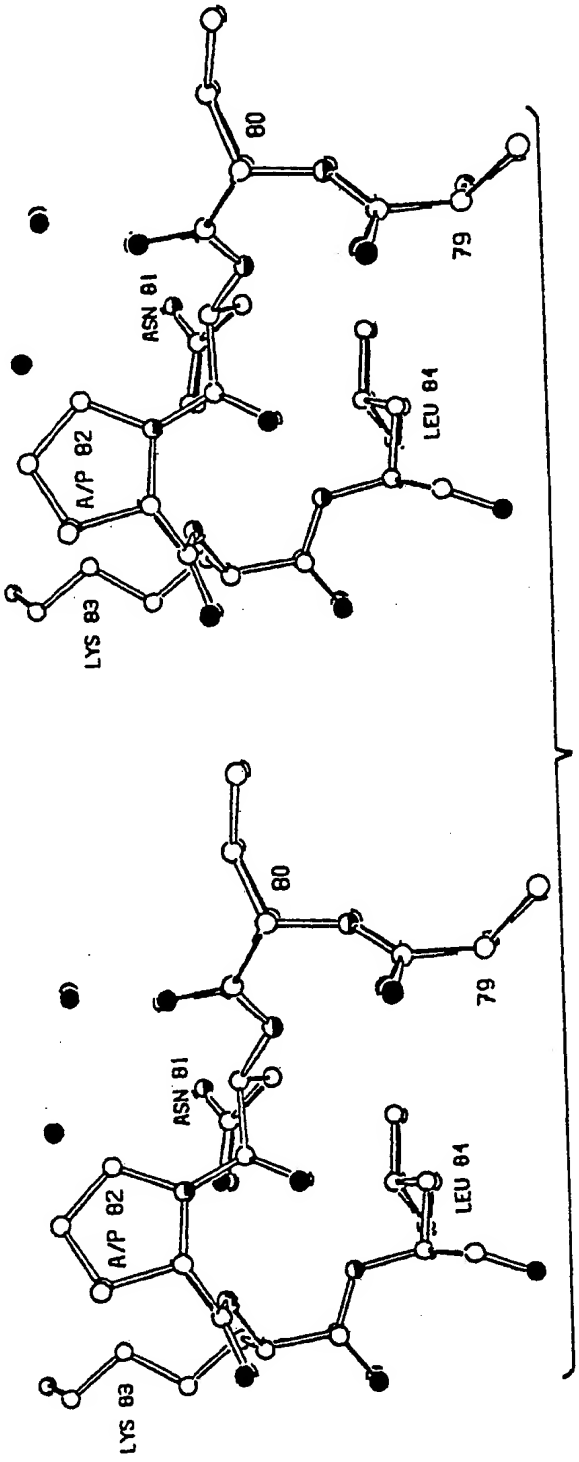


FIG. 2B

FIG. 3A

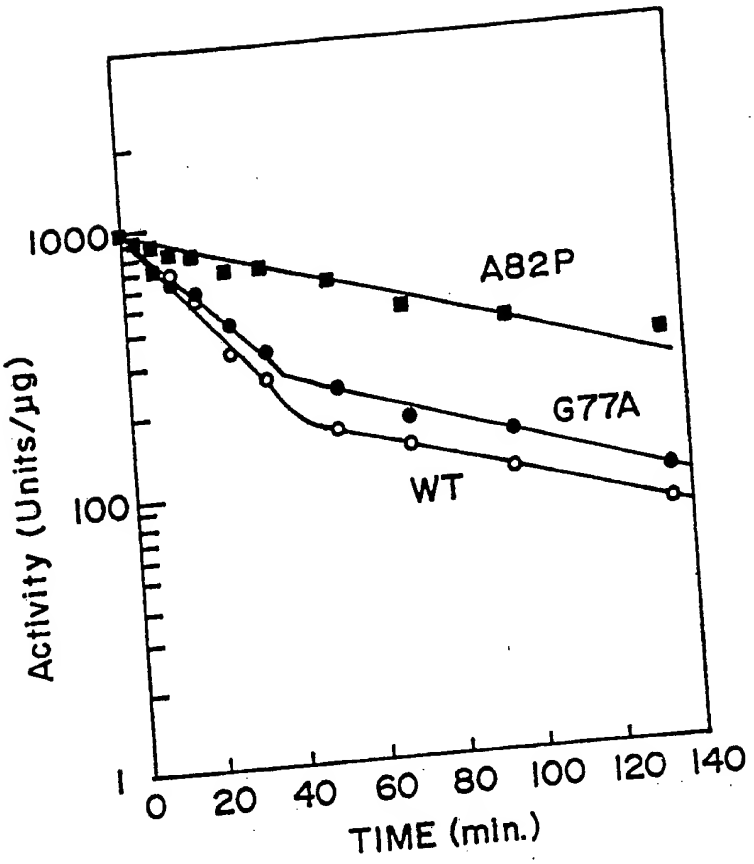
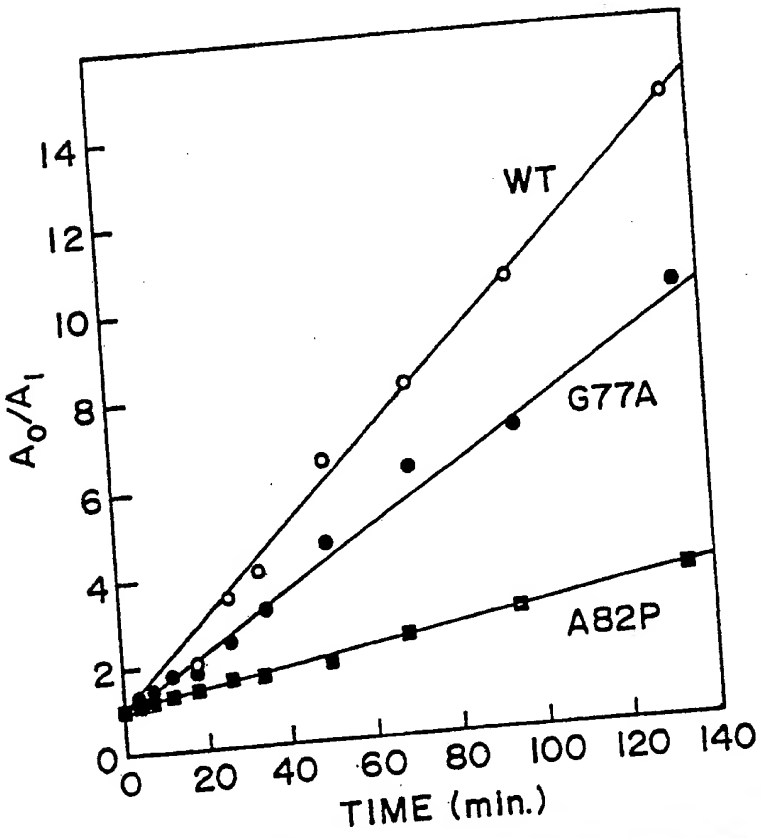

1000
100
1
Activity (Units/µg)
A82P
G77A
WT
0
20
40
60
80
100
120
140
TIME (min.)

FIG. 3B


14
12
10
8
6
4
2
0
A_0/A_1
WT
G77A
A82P
0
20
40
60
80
100
120
140
TIME (min.)

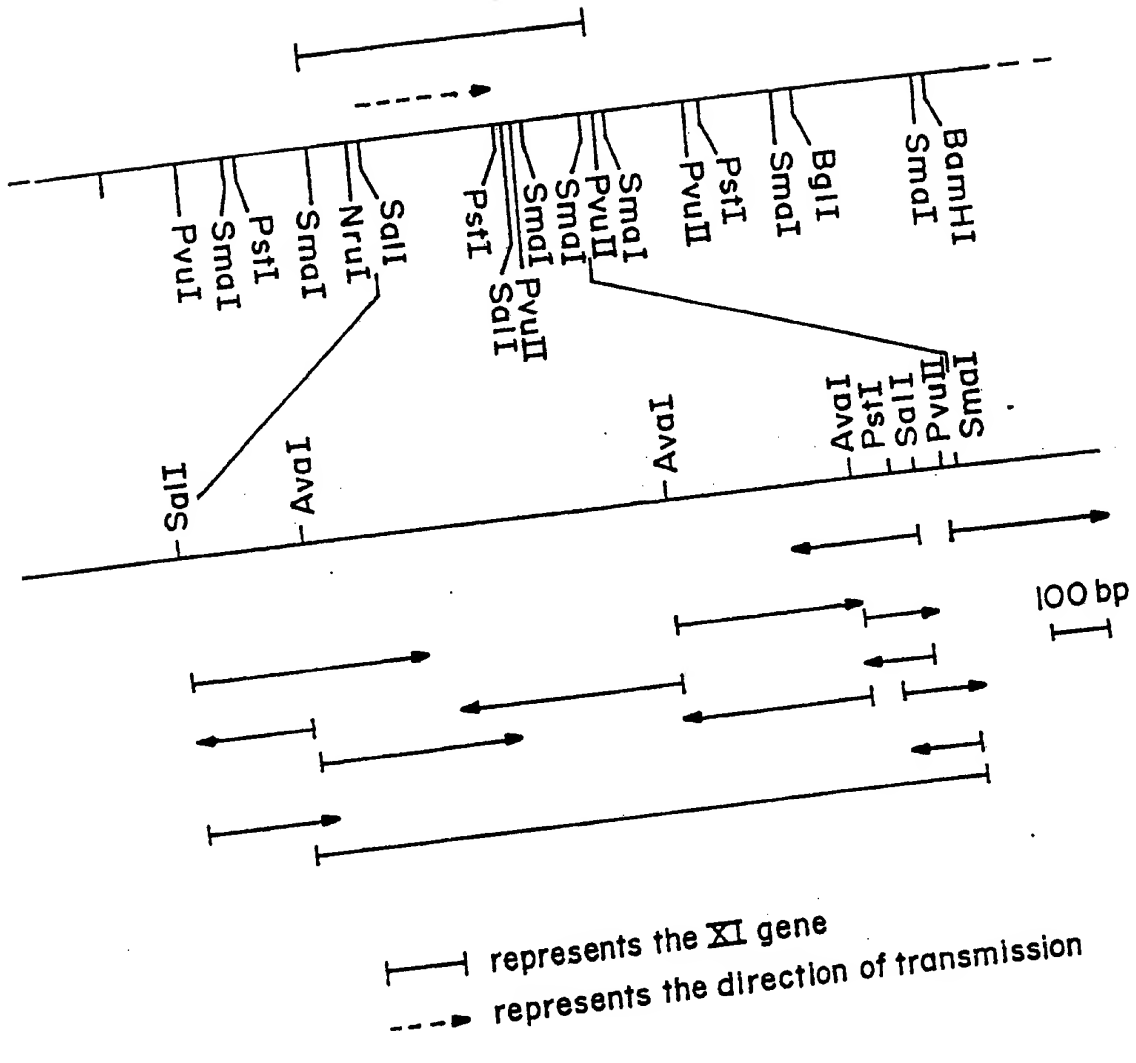

PvuI
SmaI
PstI
SmaI
NruI
SalI
PstI
SalI
PvuII
SmaI
SmaI
PvuII
PvuII
PstI
SmaI
BglI
SmaI
BamHI
SalI
AvaI
AvaI
AvaI
PstI
SalI
PvuII
SmaI
100 bp
represents the XI gene
represents the direction of transmission

FIG. 4

```
                ATGAACTACCAGCCCACCCCCGAGGACAGGTTCACCTTCGGACTGTGGACCGTCGGCTGG
1               METAsnTyrGlnProThrProGluAspArgPheThrPheGlyLeuTrpThrValGlyTrp
                CAGGGACGGGACCCCTTCGGTGACGCCACGCGGCGCGCCCTCGACCCGGTCGAGTCGGTG
21              GlnGlyArgAspProPheGlyAspAlaThrArgArgAlaLeuAspProValGluSerVal
                CGGCGGCTGGCCGAGCTGGGCGCCCACGGCGTCACGTTCCACGACGACGACCTCATCCCC
41              ArgArgLeuAlaGluLeuGlyAlaHisGlyValThrPheHisAspAspAspLeuIlePro
                TTCGGCTCCAGCGACAGCGAGCGCGAGGAGCACGTCAAGCGGTTCCGGCAGGCGCTGGAC
61              PheGlySerSerAspSerGluArgGluGluHisValLysArgPheArgGlnAlaLeuAsp
                GACACCGGCATGAAGGTGCCGATGGCCACCACCAACCTGTTCACCCACCCGGTGTTCAAG
81              AspThrGlyMETLysValProMETAlaThrThrAsnLeuPheThrHisProValPheLys
                GACGGCGGCTTCACCGCCAACGACCGCGACGTGCGCCGCTACGCCCTGCGCAAGACCATC
101             AspGlyGlyPheThrAlaAsnAspArgAspValArgArgTyrAlaLeuArgLysThrIle
                CGCAACATCGACCTCGCGGTCGAGCTCGGCGCCGAGACCTATGTGGCCTGGGGCGGCCGC
121             ArgAsnIleAspLeuAlaValGluLeuGlyAlaGluThrTyrValAlaTrpGlyGlyArg
                GAGGGTGCCGAGTCGGGTGGCGCCAAGGACGTGCGGGACGCCCTCGACCGCATGAAGGAG
141             GluGlyAlaGluSerGlyGlyAlaLysAspValArgAspAlaLeuAspArgMETLysGlu
                GCCTTCGACCTGCTCGGCGAGTACGTCACCTCCCAGGGCTACGACATCCGCTTCGCCATC
161             AlaPheAspLeuLeuGlyGluTyrValThrSerGlnGlyTyrAspIleArgPheAlaIle
                GAGCCCAAGCCGAACGAGCCGCGCGGCGACATCCTGCTCCCCACCGTCGGCCACGCCCTG
181             GluProLysProAsnGluProArgGlyAspIleLeuLeuProThrValGlyHisAlaLeu
                GCGTTCATCGAGCGCCTGGAGCGACCGGAGCTGTACGGCGTGAACCCCGAGGTCGGCCAC
201             AlaPheIleGluArgLeuGluArgProGluLeuTyrGlyValAsnProGluValGlyHis
                GAGCAGATGGCCGGGCTGAACTTCCCGCACGGCATCGCGCAGGCGCTGTGGGCGGGCAAG
```

FIG. 5A

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221 GluGlnMETAlaGlyLeuAsnPheProHisGlyIleAlaGlnAlaLeuTrpAlaGlyLys

    CTGTTCCACATCGACCTCAACGGCCAGAACGGCATCAAGTACGACCAGGACCTCCGCTTC

241 LeuPheHisIleAspLeuAsnGlyGlnAsnGlyIleLysTyrAspGlnAspLeuArgPhe

    GGCGCGGGCGACCTGCGGGCCGCGTTCTGGCTGGTGGACCTGCTGGAGTCGGCCGGCTAC

261 GlyAlaGlyAspLeuArgAlaAlaPheTrpLeuValAspLeuLeuGluSerAlaGlyTyr

    AGCGGCCCGCGGCACTTCGACTTCAAGCCGCCGCGGACCGAGGACTTCGACGGGGTGTGG

281 SerGlyProArgHisPheAspPheLysProProArgThrGluAspPheAspGlyValTrp

    GCCTCGGCGGCCGGCTGCATGCGCAACTACCTGATCCTCAAGGAGCGTGCGGCGGCCTTC

301 AlaSerAlaAlaGlyCysMETArgAsnTyrLeuIleLeuLysGluArgAlaAlaAlaPhe

    CGCGCCGACCCCGAGGTGCAGGAGGCGCTGCGCGCGTCCCGTCTGGACGAGCTGGCCCGG

321 ArgAlaAspProGluValGlnGluAlaLeuArgAlaSerArgLeuAspGluLeuAlaArg

    CCCACGGCGGCCGACGGTCTGCAGGCCCTGCTCGACGACCGGTCCGCCTTCGAGGAGTTC

341 ProThrAlaAlaAspGlyLeuGlnAlaLeuLeuAspAspArgSerAlaPheGluGluPhe

    GACGTCGACGCGGCGGCGGCCCGTGGGATGGCCTTCGAGCGCCTGGACCAGCTGGCGATG

361 AspValAspAlaAlaAlaAlaArgGlyMETAlaPheGluArgLeuAspGlnLeuAlaMET

    GACCACCTGCTGGGCGCCCGGGGCTGA

381 AspHisLeuLeuGlyAlaArgGly...
```

FIG. 5B

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FIG. 6-I

```
GI      - S. rubiginosus Glucose Isomerase (1-)
GI      - B. subtilis Glucose Isomerase (1-)
p GI    - A. spheroides Glucose Isomerase (1-)
GI      - E. coli Glucose Isomerase (1-)

GI      M N Y Q P T P E D R F T F G L W T V G W Q G R D P F G D A T R R A L D P V E S V R R - - - - - - - - L A E L G A
GI      M S L Q A T P D D K F S F G L W T V G W Q A R D A F G D A T R P V L D P I E A V H K - - - - - - - - L A E I G A
p GI    - M A Q S H S S S V N Y F G S V N K V V F E G K A S T N P L A F K Y Y N P Q E V I G G K T M K E H L R F S I A
GI      - - - - - - - - M Q A Y F D Q L D R V R Y E G S K S S N P L A F R H Y N P D E L V L G K R M E E H L R F A A C
mmon                Q               F                                                           L

GI      H G V T F H D D D L I P F G S S D S E R - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - Y E
p GI    Y G V T F H D D D L V P F G A D A A T R - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - D G
GI      Y W H T F T A D G T D V F G A A T M Q R P W D H Y K G M D L A R A R V E A - - A F E M F E K L D A P F F A F H D R
GI      Y W H T F C W N G A D M F G V G A F N R P W Q Q P - G E A L A L A K R K A D V A F E F F H K L H V P F Y C F H D V
mmon          T F                 F G         R

GI      H V K R F R Q A L D E T G M - - - - K V P M A - - - - - - - - - - - - - - - T T N L F T H P V F K D G G F T A N
p GI    I V A G F S K A L D E T G L - - - - I V P M V - - - - - - - - - - - - - - - T T N L F T H P V F K D G G F T S N
GI      D I A P E G S T L K E T N Q N L D I I V G M I K D Y M R D S N V K L L W N T A N M F T N P R F V H G A A T S C
GI      D V S P E G A S L K E Y I N N F A Q M V D V L A G K Q E E S G V K L L W G T A N C F T N P R Y G A G A A T N P
mmon                    L   E               V                           T   N   F T     P         G     T

GI      D R D V R R Y A L R K T I R N I D L A V E L G A E T Y V A W G G R E G A E S G G A K D V R D A L D R M K E A F
p GI    D R S V R R Y A I R K V L R Q M D L G A E L G A K T L V L W G G R E G A E Y D S A K D V G A A L D R Y R E A L
GI      N A D V F A Y A A A Q V K K G L E T A K E L G A E N Y V F W G G R E G Y E T L L N T D L K F E L D N L A R F M
GI      D P E V F S W A A T Q V V T A M E A T H K L G G E N Y V L W G G R E G Y E T L L N T D L R Q E R E Q L G R F M
mmon        V         A                     L G         V   W G G R E G   E             D

GI      D L L G E Y V T S Q G Y D I R F A I E P K P N E P R G D I L L P T V G H A L A F I E R L E R P E L Y G V N P E
p GI    N L L A Q Y S E D Q G Y G L P F A I E P K P N E P R G D I L L P T A G H A I A F V Q E L E R P E L F G I N P E
GI      H M A V D Y A K E I E Y T G Q F L I E P K P K E P T T H Q Y D T D A A T T I A F L K Q Y G L D N H F K L N L E
GI      Q M V V E H K H K I G F Q G T L L I E P K P Q E P T K H Q Y D Y D A A T V Y G F L K Q F G L E K E I K L N I E
mmon                                I E P K P   E P                         F                         N   E
```

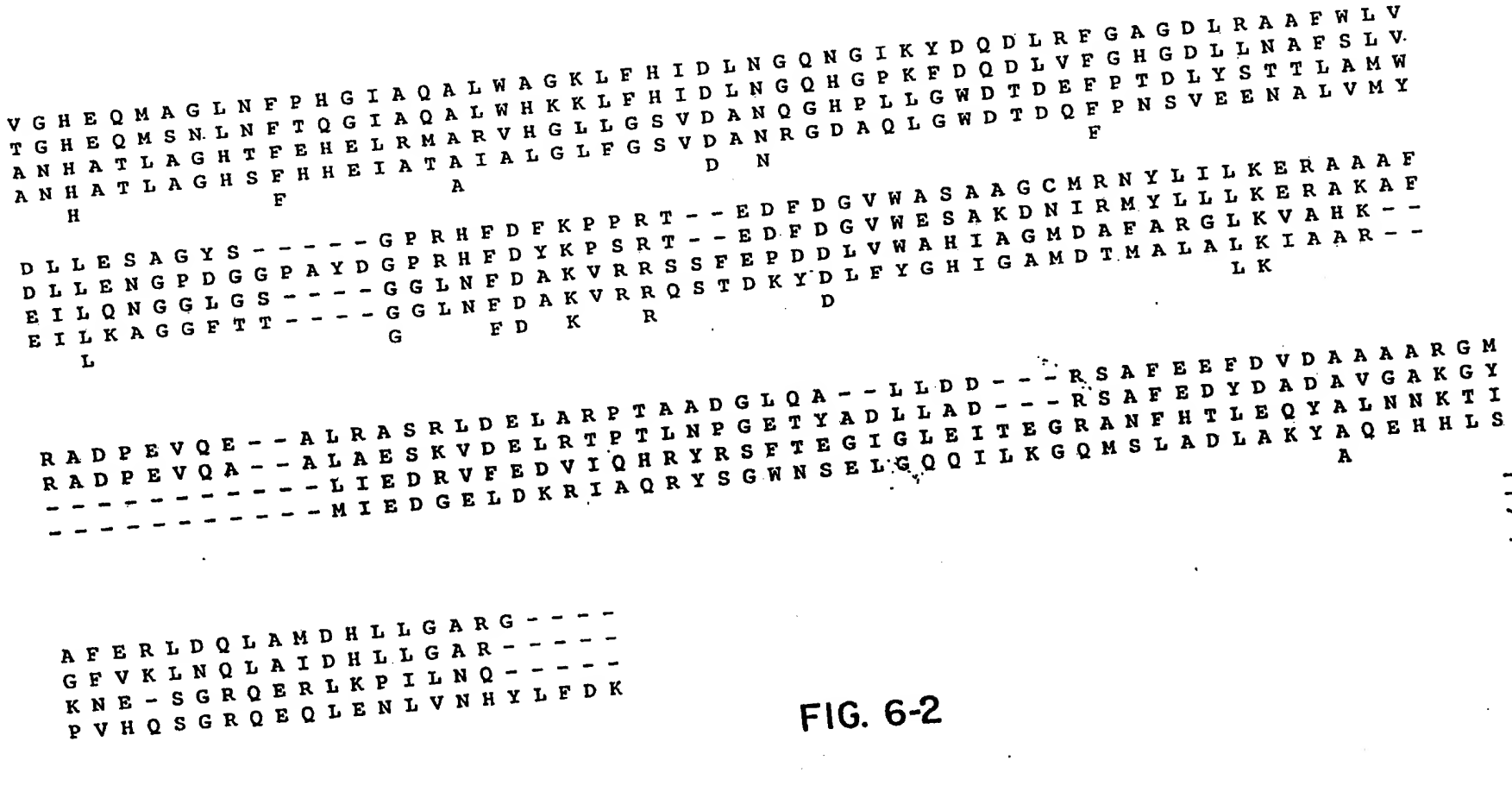


FIG. 6-2

neim et al., Nuc. Acids Res. 13: 5717-5722 (1985);

ri et al., J. Bacter. 169: 612-618 (1987);

lis et al., Appl. and Env. Biol. 47: 15-21 (1984).

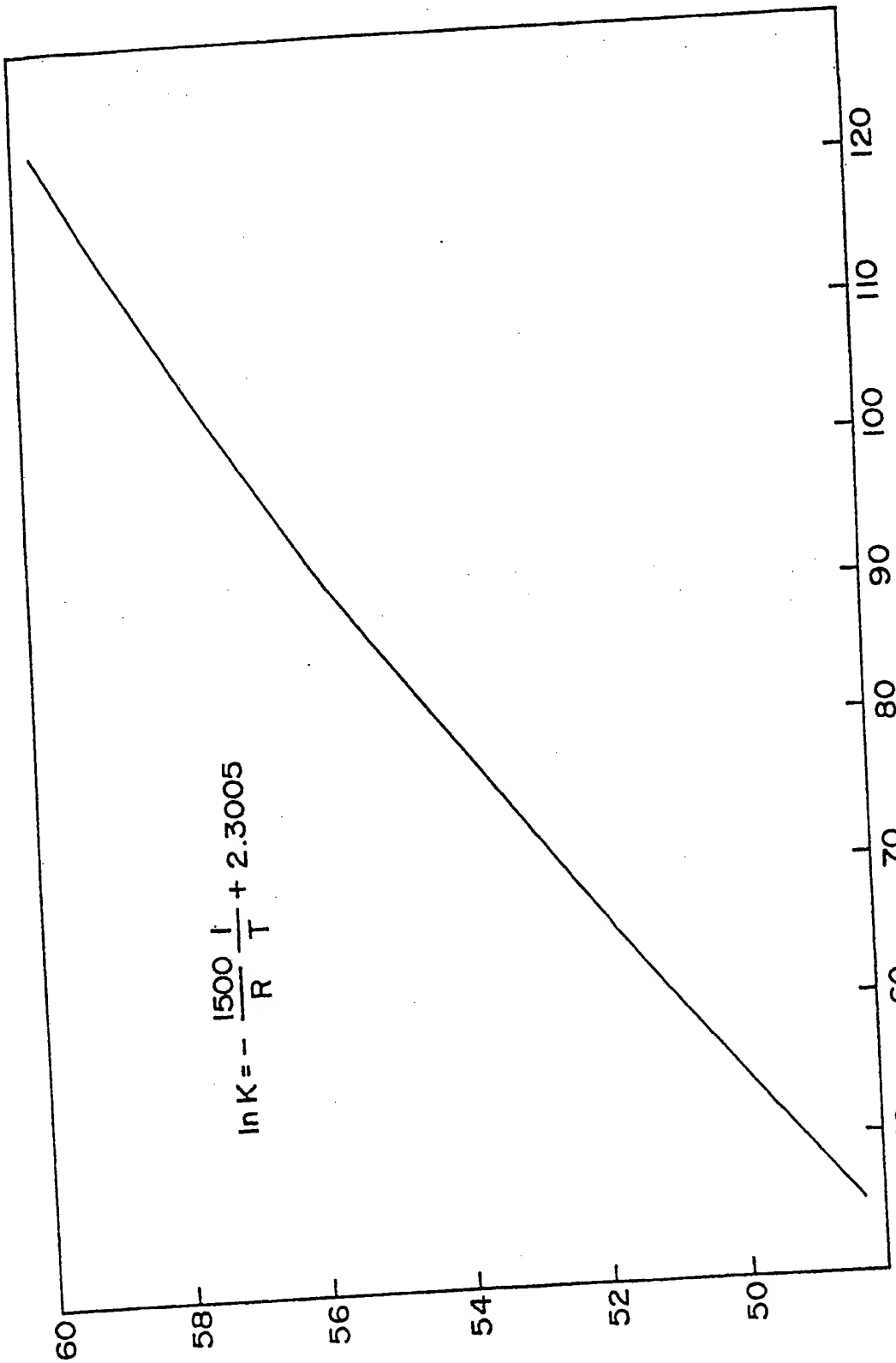
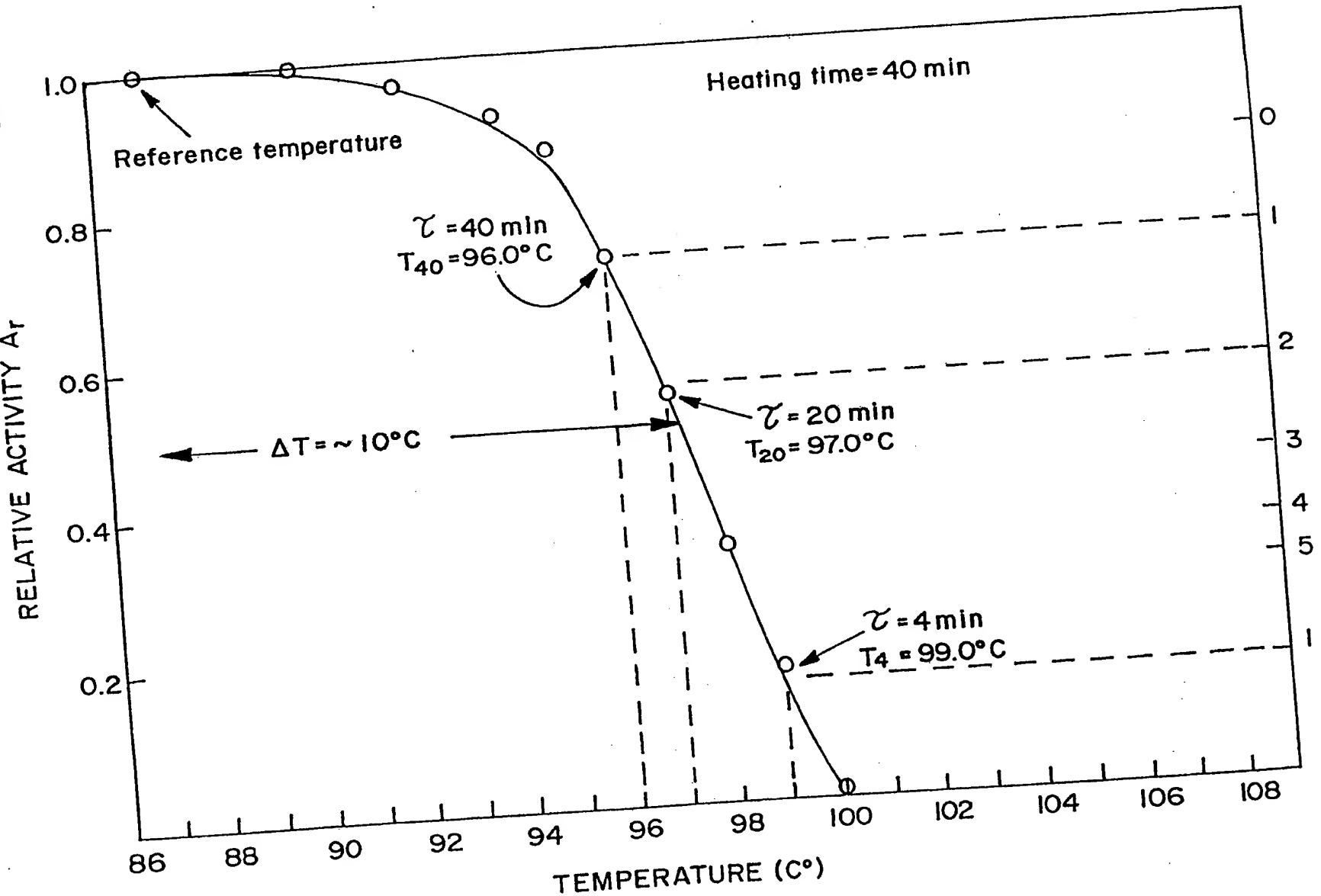

60
58
56
54
52
50
50
60
70
80
90
100
110
120
TEMPERATURE (0°C)
$\ln K = -\frac{1500}{R}\frac{1}{T} + 2.3005$

FIG. 7

FIG. 8


Heating time=40 min
Reference temperature
τ =40 min
T40=96.0°C
τ = 20 min
T20= 97.0°C
ΔT= ~ 10°C
τ = 4 min
T4 = 99.0°C
RELATIVE ACTIVITY Ar
1.0
0.8
0.6
0.4
0.2
86
88
90
92
94
96
98
100
102
104
106
108
TEMPERATURE (C°)
0
1
2
3
4
5
1

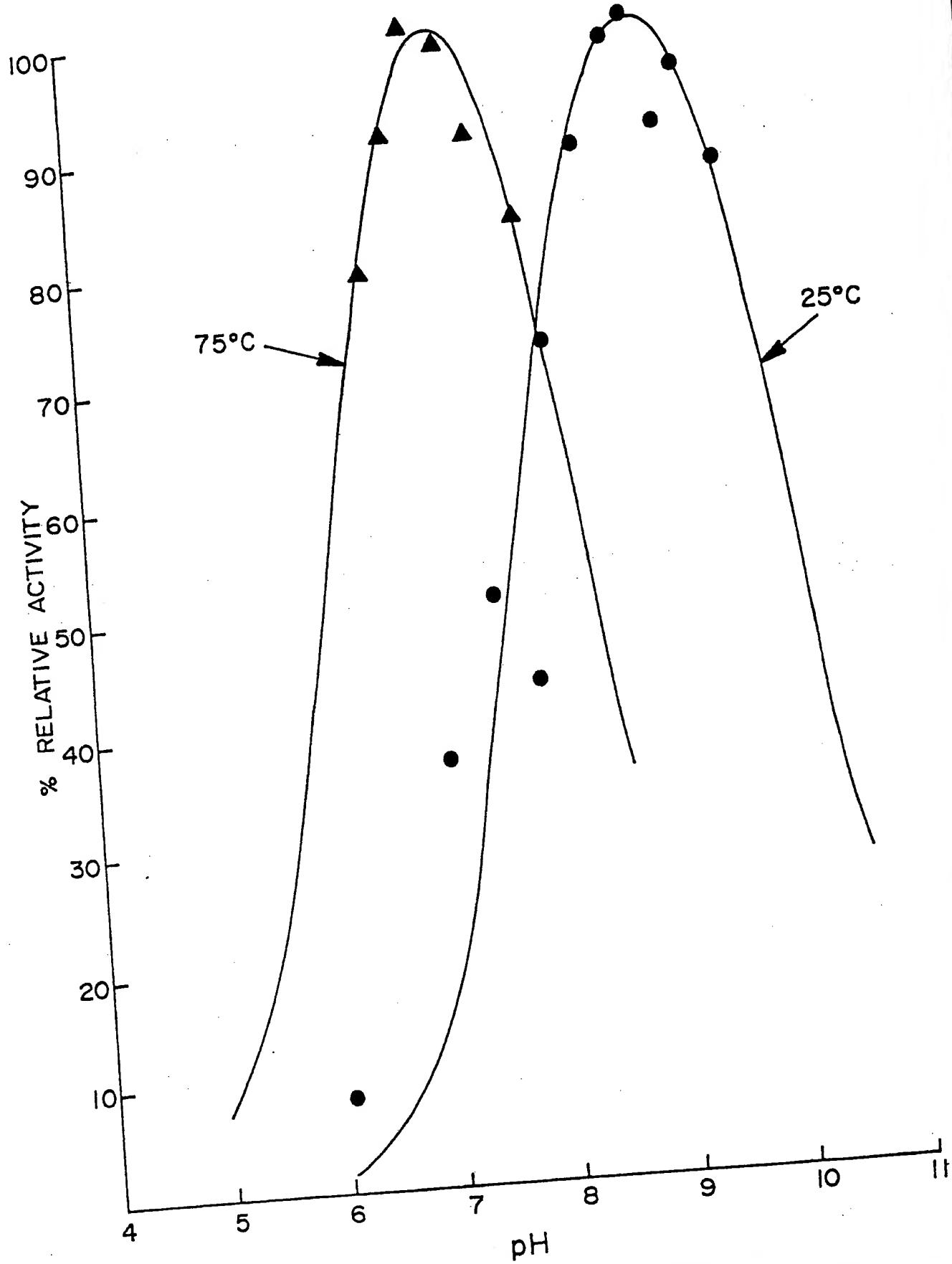

100
90
80
70
60
50
40
30
20
10
% RELATIVE ACTIVITY
75°C
25°C
4
5
6
7
8
9
10
11
pH

FIG 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/02765

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00, C 12 N 9/92, C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N, C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Chemical Abstracts, volume 106, no. 15, 13 April 1987, (Columbus, Ohio, US), Snow,Mark E. et al: "Calculating three-dimensional changes in protein structure due to amino-acid substitutions; the variable region of immunoglobulins", see page 459, abstract 117680v, & Proteins: Struct.,Funct., Genet.1986, 1(3), 267-79 (Eng).	1-6
Y	Proc.Natl.Acad.Sci. USA, volume 84, pp 6663-6667, October 1987, Biochemistry B.W. Matthews et al: "Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding", see abstract	1-6,7-8-12
Y	Nature, volume 307, 12 January 1984,pp 187-188 Anthony J. Wilkinson et al: "A large increase in enzyme-substrate affinity -/-	1-6

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1st December 1988	28 DEC 1988
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	by protein engineering" see abstract	
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Y	Chemical Abstracts, volume 90, no. 9, 26 February 1979, (Columbus,Ohio,US), Creighton, Thomas E.: "Possible implications of many proline residues for the kinetics of protein unfolding and refolding", see page 160, abstract 68016y, & J.Mol.Biol. 1978, 125(3), 401-6 (Eng)	1-6
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Y	Research Article, 19 April 1985, p 291 Charles S. Craik et al: "Redesigning Trypsin: Alteration of Substrate Specificity", see whole document,	1-6 7-8,12
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Y	US, A, 4 410 627 (NORMAN E. LLOYD et al) 18 October 1983 see column 7, lines 51-52	7-12
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Y	Chemical Abstracts, volume 100, no. 17, 23 April 1984, (Columbus, Ohio, US), Carrell, H.L. et al: "X-ray crystal structure of D-xylose isomerase at 4-Å resolution", abstract 135020k, & J. Biol. Chem. 1984, 259(5), 3230-6 (Eng)	7-12
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Y	Chemical Abstracts, volume 108, no. 15, 11 April 1988, (Columbus, Ohio, US), Farber, Gregory K et al: "The 3.0 A crystal structure of xylose isomerase from Streptomyces olivochromogenes", abstract 127608h, & Protein Eng. 1987, 1(6), 459-66 (Eng)	7-12
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Y	Chemical Abstract, volume 108, no. 17, 25 April 1988, (Columbus, Ohio, US), Henrick, K. et al: "Comparison of backbone structures of glucose isomerase from Streptomyces and Arthrobacter", abstract 146202n, & Protein Eng. 1987, 1(6), 467-9 (Eng)	7-12
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US88/02765 SA 24149

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/11/88
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4410627	18-10-83	BE-A- 897165	29-12-83
		GB-A- 2123001	25-01-84
		FR-A- 2529572	06-01-84
		SE-A- 8303725	31-12-83
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		NL-A- 8302334	16-01-84
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		CA-A- 1200521	11-02-86
		AU-A- 558696	05-02-87
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		US-A- 4393137	12-07-83

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A2, 0 068 647 (THE UPJOHN COMPANY) 5 January 1983 whole document	7-12
